International Journal of Food Microbiology 132 (2009) 134–140

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Efficacy of chlorine, acidic electrolyzed water and aqueous chlorine dioxide solutions to decontaminate *Escherichia coli* O157:H7 from lettuce leaves ☆

Lindsey A. Keskinen, Angela Burke, Bassam A. Annous *

Food Safety Intervention Technologies Research Unit, U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, PA 19038-8598, United States

ARTICLE INFO

Article history:
Received 24 November 2008
Received in revised form 6 April 2009
Accepted 10 April 2009

Keywords:
Escherichia coli O157:H7
Lettuce
Leafy greens
Sanitizer
Chlorine dioxide
Chlorine
Acidified sodium chlorite
Acidic electrolyzed water

ABSTRACT

This study compared the efficacy of chlorine (20–200 ppm), acidic electrolyzed water (50 ppm chlorine, pH 2.6), acidified sodium chlorite (20–200 ppm chlorite ion concentration, Sanova®), and aqueous chlorine dioxide (20–200 ppm chlorite ion concentration, TriNova®) washes in reducing populations of *Escherichia coli* O157:H7 on artificially inoculated lettuce. Fresh-cut leaves of Romaine or Iceberg lettuce were inoculated by immersion in water containing *E. coli* O157:H7 (8 log CFU/ml) for 5 min and dried in a salad spinner. Leaves (25 g) were then washed for 2 min, immediately or following 24 h of storage at 4 °C. The washing treatments containing chlorite ion concentrations of 100 and 200 ppm were the most effective against *E. coli* O157:H7 populations on Iceberg lettuce, with log reductions as high as 1.25 log CFU/g and 1.05 log CFU/g for TriNova® and Sanova® wash treatments, respectively. All other wash treatments resulted in population reductions of less than 1 log CFU/g. Chlorine (200 ppm), TriNova®, Sanova®, and acidic electrolyzed water were all equally effective against *E. coli* O157:H7 on Romaine, with log reductions of ~1 log CFU/g. The 20 ppm chlorine wash was as effective as the deionized water wash in reducing populations of *E. coli* O157:H7 on Romaine and Iceberg lettuce. Scanning electron microscopy indicated that *E. coli* O157:H7 that was incorporated into biofilms or located in damage lettuce tissue remained on the lettuce leaf, while individual cells on undamaged leaf surfaces were more likely to be washed away.

Published by Elsevier B.V.

1. Introduction

Escherichia coli O157:H7 has been implicated in a number of recent recalls and outbreaks of illness linked to the consumption of raw leafy green vegetables, both in the United States and internationally (CDPH, 1996, 2002, 2004a,b, 2005, 2007a,b, 2008; Hilborn et al., 1999; MMWR, 2006; Soderstrom et al., 2008). A number of these outbreaks have been linked to packaged, pre-washed, ready-to-eat leafy greens, while recalled products include prewashed baby spinach (CDPH, 2004a, 2007a; MMWR, 2006), shredded ready-to-eat Iceberg lettuce (CDPH, 2007b, 2008), pre-packaged, ready-to-eat salads containing Romaine lettuce (CDPH, 2002, 2005), and salad mixes containing Iceberg, Romaine, and other leafy greens (CDPH, 1996, 2004b; Hilborn et al., 1999).

Since leafy green vegetables are consumed raw, sanitizing washes constitute the most practical means of decontamination of these products. In commercial value-added produce processing, chlorine rinses are frequently used with concentrations varying from 50 to 200 ppm and with contact times seldom exceeding 2 min (Parish et al., 2003). Although chlorine is the most commonly used sanitizer, it is inactivated by organic material and can also lead to the formation of potentially carcinogenic and teratogenic trihalomethanes and haloacetic acids (Stevens, 1982). However, the benefits of chlorine use for the produce industry outweigh the concerns of potential formation of harmful byproducts. Studies have shown that chlorine rinses can decrease the bacterial load by values ranging from <1 log CFU/g to 3.15 log CFU/g (Akbas and Olmez, 2006; Beuchat, 1999; Beuchat et al., 2004; Burnett et al., 2004; Escudero et al., 1999; Nthenge et al., 2007), depending on inoculation method, chlorine concentration, contact time, and the target bacteria tested. Although the antimicrobial efficacy of chlorine rinses may be different for different lettuce varieties, the log reductions achieved in most studies were equivalent to those caused by water wash treatments (Beuchat, 1999; Nthenge et al., 2007); although this could differ between varieties of lettuce (Beuchat et al., 2004; Burnett et al., 2004).

A concern by the produce industry for the potential regulatory constraints on using chlorine in its present form has increased efforts to identify and evaluate alternative sanitation agents. Acidic electrolyzed water (AEW) has been marketed (Hoshizaki Electric Co., Ltd., 2003) as more effective than chlorine rinses due to a combination of low pH (2.6), high oxidation reduction potential (+1200 mV), and low residual chlorine concentration. Major advantages of using AEW over sodium hypochlorite are: 1) AEW is produced on site by the electrolysis of

☆ Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

* Corresponding author. Tel.: +1 215 233 6797; fax: +1 215 233 6406.
E-mail address: bassam.annous@ars.usda.gov (B.A. Annous).

0168-1605/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.ijfoodmicro.2009.04.006

sodium chloride solution with the help of an electrolysis flow generator, and 2) there is no need for handling or storage of potentially dangerous sodium hypochlorite in liquid or solid form (Kim et al., 2003). When evaluated against *E. coli* O157:H7, *Salmonella enteritidis*, and *Listeria monocytogenes* in a pure culture suspensions, AEW was found to reduce populations by 7 log CFU/ml within 5 min, and to eliminate the pathogens completely within 10 min (Venkitanarayanan et al., 1999). However, when evaluated for its efficacy in reducing *E. coli* O157:H7 populations on lettuce, AEW has been found to achieve reductions ranging from <1 log CFU/g to 4.6 log CFU/g, depending on inoculation method, treatment temperature and chlorine concentration, with spray- and spot-inoculation frequently resulting in greater reported log reductions by AEW and other sanitizers, as compared to dip-inoculation (Koseki et al., 2003; Park et al., 2008; Stopforth et al., 2008). In none of these cases, however, was the sanitizing solution able to completely eliminate the pathogen from lettuce (Koseki et al., 2003; Park et al., 2008; Stopforth et al., 2008).

Chlorine dioxide and acidified sodium chlorite have also attracted interest as alternatives to chlorine. Unlike chlorine, chlorine dioxide does not participate in chlorination reactions that result in harmful byproducts. Rodgers et al. (2004) found that aqueous chlorine dioxide (5 ppm) was able to achieve greater than 5 log reductions of *L. monocytogenes* and *E. coli* O157:H7 on apples, lettuce and cantaloupe. These types of results are highly dependent on the method used to inoculate the produce—other studies have found that the same level of aqueous chlorine dioxide was only capable of reducing *L. monocytogenes* on lettuce by 1.7 log CFU/g (Zhang and Farber, 1996). Acidified sodium chlorite has been used at various concentrations on fresh cut produce, and complete inactivation of *E. coli* O157:H7 has been documented on carrots washed with 1000 ppm of the compound (Gonzalez et al., 2004). Another study reported that a commercial brand of acidified sodium chlorite used at concentrations of 250–500 ppm was not significantly different from water in its ability to reduce coliforms on lettuce (Allende et al., 2008). A recent study has reported that acidified sodium chlorite at 1200 ppm was capable of reducing *E. coli* O157:H7 and *Salmonella* on spray-inoculated lettuce by more than 3 log CFU/g (Stopforth et al., 2008).

Studies reported in the literature present differences regarding lettuce variety, inoculation and recovery methods, standardization of active ingredients' concentrations in treatment solutions, and treatment times and conditions. These factors make it difficult to compare the relative efficacy of sanitizers against *E. coli* O157:H7 on lettuce based on currently available information. The purpose of this study was to compare the efficacy of similar concentrations of chlorine and alternative sanitizers against *E. coli* O157:H7 on two different types of lettuce: Iceberg (*Lactuca sativa* L.) and Romaine (*Lactuca sativa* L. var. *longifolia*). We also investigated the effect that bacterial attachment time and sanitizing treatment times may have on *E. coli* O157:H7 survival.

2. Materials and methods

2.1. Bacterial strain and inoculum preparation

E. coli O157: H7 SEA13B88 (human feces, apple cider-associated disease outbreak), maintained at −80 °C in trypticase soy broth (TSB; Becton Dickinson, Sparks, MD) and 10% (v/v) glycerol, was grown for 18–24 h in TSB at 37 °C, transferred to a trypticase soy agar (TSA; Becton Dickinson) slant, and this working stock culture was stored at 4 °C for no more than 21 d. Inoculum was prepared by transferring a loopful (1 µl) of the working stock to 10 ml TSB, which was incubated in a shaking incubator for 6–8 h at 37 °C. Following incubation, 180 µl of the culture was transferred to 1.8 L of TSB, and then incubated at 37 °C in a shaking incubator for 18–24 h. The overnight culture was then centrifuged (6740 ×*g*) at 4 °C for 15 min. After decanting the supernatant, the resulting pellet was resuspended in sterile deionized water and centrifuged (6740 ×*g*) for 15 min at 4 °C. The supernatant was decanted and the pellet was resuspended in 3.6 L of sterile deionized water. The concentration of the inoculum was determined by serially diluting the inoculum in 0.1% peptone water (PW; Becton Dickinson) and plating on TSA.

2.2. Dip inoculation of lettuce

Commercially available Iceberg lettuce (*Lactuca sativa*) and Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) were purchased at a local supermarket, and stored at 4±2 °C for a maximum of 24 h before use in experiments. Damaged outer leaves were removed from each head of lettuce, the lettuce was cut into pieces approximately 4–6 cm^2 and immediately submerged into the *E. coli* O157:H7 inoculum suspension for 5 min. Excess liquid culture on the lettuce was removed using a salad spinner (OXO Good Grips Salad Spinner, OXO International, Ltd., New York, NY) for 1 min and then the lettuce was placed into an open container and allowed to dry for 2 h at 22±2 °C in a biosafety cabinet. After 2 h, a subsample of the inoculated lettuce was analyzed to determine the initial *E. coli* O157:H7 concentration, as described below. The remainder of the inoculated samples were then divided into two sets: those that were treated immediately (day 0), or placed into plastic food storage bags and stored aerobically for 18–24 h at 4±2 °C prior to treatment (day 1).

2.3. Preparation of treatment solutions

Chlorine solutions (500 ml) were made immediately before use by diluting 6.0% sodium hypochlorite (Clorox, The Clorox Company, Oakland, CA) in deionized water to achieve concentrations of 20, 100 or 200 ppm chlorine. Concentrations were verified with chlorine concentration test strips (*Advantec* MHS, Inc., Dublin, CA). Acidic electrolyzed water (50 ppm chlorine, pH 2.6, +1200 mV) was generated using a Hoshizaki Water Electrolyzer (Model ROX-20TA-U; Hoshizaki Electric Co., Ltd., Griffin, GA) with a 13% (w/v) sodium chloride solution to obtain 500 ml of acidic electrolyzed water, which was used within 4 h of generation. An aqueous chlorine dioxide stock solution was made by using chlorine dioxide sachets (Tri-Nova® 2-g solution pack, ICA Tri-Nova, LLC, Newnan, GA), mixed according to manufacturer instructions, in a sealed bottle of deionized water (1 L) and allowing the reaction to go to completion for at least 3 d at 22± 2 °C, in the absence of light. The chlorite ion concentration was then measured by titration (Titralab Model TIM840; Radiometer Analytical SAS, Villeurbanne CEDEX, France) immediately before use, and diluted with deionized water to make 500 ml solutions containing 20, 100, and 200 ppm chlorite ion. Sanova®, a commercially available brand of acidified sodium chlorite (Ecolab, St. Paul, MN) was prepared by mixing citric acid and sodium chlorite solutions. The solutions were mixed according to manufacturer directions to give 1000 ppm Sanova®, and stored in a sealed 1 L bottle at 22±2 °C in the absence of light, for either 30 min or 18–24 h. Chlorous acid is the predominant form of the chlorite ion in the Sanova® solution at pH 2.3–3.2, and is considered stable for the first 30 min after combining the acid and sodium chlorite (Kross and Kemp, 2000). On the other hand, chlorine dioxide is the predominant form of chlorite ion when the reaction is allowed to go to completion for at least 18–24 h. The chlorite ion concentration was measured via titration and adjusted to 20, 100 and 200 ppm for Sanova® allowed to react for 18–24 h, or 100 ppm for Sanova® allowed to react for 30 min, by diluting in deionized water immediately before using to treat lettuce. All chlorine and chlorite solutions were used immediately following preparation and treatments were conducted in chlorine-aged glassware at 22±2 °C.

2.4. Sanitizing treatment of lettuce

Inoculated Iceberg or Romaine lettuce portions (25 g) were stirred into 500 ml of sanitizer solution and incubated at 22±2 °C for 2 min

Table 1
Efficacy of various sanitizer treatments (2 min) on reducing *Escherichia coli* O157:H7 populations on artificially inoculated Iceberg lettuce (*Lactuca sativa* L.) stored at 4 °C for 18–24 h.

Treatment[a]	CTSMAC (log CFU/g)		Recovery (log CFU/g)		Percent injury
	Population[b]	Mean reduction	Population[b]	Mean reduction	
Untreated control	7.19 ± 0.48^{a}		7.77 ± 0.20^{a}		73.7
Deionized water, pH 7.0	6.64 ± 0.55^{ab}	0.55	7.38 ± 0.20^{b}	0.39	81.8
Chlorine, 20 ppm, pH 8.0	6.38 ± 0.28^{abc}	0.81	7.21 ± 0.01^{bc}	0.56	85.2
Chlorine, 200 ppm, pH 8.0	6.53 ± 0.33^{abc}	0.66	7.11 ± 0.07^{bc}	0.66	73.7
TriNova, 20 ppm ClO_2^-, pH 8.0	6.13 ± 0.42^{bc}	1.06	6.94 ± 0.21^{cdef}	0.83	84.5
TriNova, 100 ppm ClO_2^-, pH 8.0	6.08 ± 0.29^{bc}	1.11	6.75 ± 0.18^{defg}	1.02	78.6
TriNova, 200 ppm ClO_2^-, pH 8.0	5.74 ± 0.22^{c}	1.45	6.34 ± 0.18^{g}	1.43	83.4
Sanova, 20 ppm ClO_2^-, pH 2.6	6.15 ± 0.97^{bc}	1.04	6.98 ± 0.23^{cdef}	0.79	85.2
Sanova, 100 ppm ClO_2^-, pH 2.6	6.00 ± 0.88^{bc}	1.19	6.72 ± 0.23^{efg}	1.05	80.9
Sanova, 200 ppm ClO_2^-, pH 2.6	6.04 ± 0.96^{bc}	1.15	6.55 ± 0.29^{defg}	1.22	80.0
Sanova, 30 min, 100 ppm ClO_2^-, pH 2.6	6.71 ± 0.66^{ab}	0.48	6.69 ± 0.07^{fg}	1.08	0.0
Acidic electrolyzed water, 50 ppm Cl_2, pH 2.6	6.51 ± 0.56^{abc}	0.68	7.05 ± 0.08^{cd}	0.72	71.2

[a] Untreated control, $n = 19$; deionized water, chlorine 20 ppm, chlorine 200 ppm, and acidic electrolyzed water, $n = 3$; TriNova and Sanova, $n = 6$.
[b] Within the same column, means not followed by the same letter are significantly different ($P < 0.05$). Data is reported as log CFU/g ± standard deviation.

for all treatments, and 10 or 20 min for AEW treatments, on an orbital shaker (70 rpm). Prior to assaying for residual *E. coli* O157:H7 cells, excess sanitizer on lettuce was removed using a salad spinner.

2.5. Microbiological analysis

Immediately following sanitizing treatments, the Iceberg or Romaine lettuce samples were diluted 1:3 (w/v) in DE Neutralizing Buffer (Becton Dickinson) and blended for 1 min in a Waring commercial blender (model 51BL31, Waring Commercial, Torrington, CT). The resulting samples were then serially diluted in PW and plated on CTSMAC, consisting of Sorbitol MacConkey Agar (Remel, Lenexa, KS) supplemented with cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L) (SMAC Media Cefixime-Tellurite Supplement; Invitrogen Dynal AS, Oslo, Norway), and incubated at 35 °C for 18–24 h. To facilitate recovery of injured cells, samples were plated on TSA, which was incubated at 35 °C for 2 h. After 2 h, the TSA plates were overlaid with CTSMAC (Recovery medium) and incubated at 35 °C for an additional 18–24 h. Following incubation of plates, colonies were enumerated and percent injury was calculated as follows:

$$\text{Percent injury} = [(\text{Recovery count} - \text{CTSMAC count}) / \text{Recovery count}] * 100$$

2.6. Scanning electron microscopy

Dip-inoculated 1–2 cm-sized samples of Romaine lettuce were stored for 18–24 h at 4 ± 2 °C, and then were fixed for scanning electron microscopy (SEM) along with samples that were washed with 20 or 200 ppm chlorine (3 samples/treatment). Samples for SEM were immersed in 20 mL aliquots of 2.5% glutaraldehyde-0.1 M imidazole buffer and sealed for 12–24 h before washing in imidazole buffer and dehydrating in 50%, 80% and absolute ethanol. Samples were then critical point dried with carbon dioxide, mounted with Duco cement (ITW Performance Polymers, Riviera Beach, FL) and colloidal silver adhesive, and then were sputter-coated with a thin layer of gold. Samples were imaged using a Quanta200 environmental scanning electron microscope (FEI Co., Inc., Hillsboro, OR), operated in the high vacuum, secondary electron imaging mode.

2.7. Statistical analysis

All sanitizer experiments were replicated three times. The resulting data were analyzed using SAS software (SAS Version 8; SAS Institute, Cary, NC) with a general linear mixed effects model and analysis of variance (ANOVA) for least significant differences among the combinations of treatments ($P < 0.05$). Means were separated by the least significant differences (LSD) test.

3. Results

3.1. Efficacy of 2 min wash treatments against Escherichia coli O157:H7 on Iceberg or Romaine lettuce

Sanitizing solutions containing aqueous chlorine dioxide or chlorous acid at 100 or 200 ppm (Sanova® and TriNova®) were more effective than other sanitizers tested against *E. coli* O157:H7 on Iceberg lettuce, achieving population reductions in excess of 1 log CFU/g (Table 1). However, 200 ppm chlorine, AEW, TriNova® and

Table 2
Efficacy of various sanitizer treatments (2 min) on reducing *Escherichia coli* O157:H7 populations on artificially inoculated Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) stored at 4 °C for 18–24 h.

Treatment[a]	CTSMAC (log CFU/g)		Recovery (log CFU/g)		Percent injury
	Population[b]	Mean reduction	Population[b]	Mean reduction	
Untreated control	7.18 ± 0.47^{ab}		7.91 ± 0.08^{a}		81.4
Deionized water, pH 7.0	6.63 ± 0.43^{bcd}	0.55	7.32 ± 0.15^{b}	0.59	79.6
Chlorine, 20 ppm, pH 8.0	6.66 ± 0.20^{bcd}	0.52	7.23 ± 0.06^{bc}	0.68	73.1
Chlorine, 200 ppm, pH 8.0	6.53 ± 0.32^{bcd}	0.65	7.00 ± 0.14^{cde}	0.91	66.1
TriNova, 20 ppm ClO_2^-, pH 8.0	6.74 ± 0.10^{bcd}	0.44	6.95 ± 0.06^{cde}	0.96	38.3
TriNova, 100 ppm ClO_2^-, pH 8.0	6.63 ± 0.19^{bcd}	0.55	6.86 ± 0.12^{de}	1.05	41.1
TriNova, 200 ppm ClO_2^-, pH 8.0	6.35 ± 0.22^{de}	0.83	6.78 ± 0.13^{e}	1.13	62.8
Sanova, 20 ppm ClO_2^-, pH 2.6	6.42 ± 0.39^{cde}	0.76	7.09 ± 0.10^{bcde}	0.82	78.6
Sanova, 100 ppm ClO_2^-, pH 2.6	6.24 ± 0.57^{de}	0.94	7.13 ± 0.46^{bcd}	0.78	87.1
Sanova, 200 ppm ClO_2^-, pH 2.6	6.70 ± 0.70^{bcd}	0.48	7.12 ± 0.09^{bcd}	0.69	62.0
Sanova, 30 min, 100 ppm ClO_2^-, pH 2.6	6.49 ± 0.03^{bcde}	0.69	7.00 ± 0.12^{cde}	0.91	68.5
Acidic electrolyzed water, 50 ppm Cl_2, pH 2.6	6.68 ± 0.34^{bcd}	0.50	7.14 ± 0.17^{bcd}	0.77	65.3

[a] Untreated Control, $n = 19$; Deionized Water, Chlorine 20 ppm, Chlorine 200 ppm, and acidic electrolyzed water, $n = 3$; TriNova and Sanova, $n = 6$.
[b] Within the same column, means not followed by the same letter are significantly different ($P < 0.05$). Data is reported as log CFU/g ± standard deviation.

Table 3
Effect of duration of attachment time (0 d vs. 1 d) of lettuce leaves inoculated with *Escherichia coli* O157:H7 prior to chlorine wash treatment (2 min) on populations of the pathogen.

Lettuce variety	Treatment	Time (d)	CTSMAC (log CFU/g)		Recovery (log CFU/g)		Percent injury
			Population[c]	Mean reduction	Population[c]	Mean reduction	
Iceberg	Untreated	0[a]	6.50 ± 0.73[abc]		8.01 ± 0.11[a]		96.9
		1[b]	7.19 ± 0.48[a]		7.77 ± 0.20[a]		73.7
	Deionized water	0[a]	6.16 ± 0.88[bc]	0.34	7.39 ± 0.06[b]	0.63	94.1
		1[a]	6.64 ± 0.55[ab]	0.55	7.38 ± 0.20[b]	0.39	81.8
	Chlorine, 20 ppm	0[a]	5.79 ± 0.97[c]	0.71	7.16 ± 0.26[bc]	0.85	95.7
		1[a]	6.38 ± 0.28[abc]	0.81	7.21 ± 0.01[bc]	0.56	85.2
	Chlorine, 200 ppm	0[a]	6.23 ± 0.43[bc]	0.27	7.01 ± 0.03[c]	1.00	83.4
		1[a]	6.53 ± 0.33[abc]	0.66	7.11 ± 0.07[bc]	0.66	73.7
Romaine	Untreated	0[a]	7.51 ± 0.48[a]		7.95 ± 0.05[a]		63.7
		1[b]	7.18 ± 0.47[ab]		7.91 ± 0.08[a]		81.4
	Deionized Water	0[a]	6.68 ± 0.42[bc]	0.83	7.05 ± 0.60[b]	0.90	57.3
		1[a]	6.63 ± 0.43[bc]	0.55	7.31 ± 0.15[b]	0.60	79.1
	Chlorine, 20 ppm	0[a]	6.13 ± 0.71 [cd]	1.37	7.16 ± 0.09[b]	0.79	90.7
		1[a]	6.66 ± 0.20[bc]	0.52	7.23 ± 0.06[b]	0.68	73.1
	Chlorine, 200 ppm	0[a]	5.92 ± 0.71[d]	1.58	6.99 ± 0.10[b]	0.96	91.5
		1[a]	6.53 ± 0.32 [cd]	0.65	7.00 ± 0.14[b]	0.91	66.1

[a] $n = 3$.
[b] $n = 19$.
[c] Within the same column, means of the same lettuce variety not followed by the same letter are significantly different ($P < 0.05$). Data is reported as log CFU/g ± standard deviation.

Sanova® treatments were equally effective in inactivating *E. coli* O157:H7 on Romaine lettuce, with population reductions not significantly greater than 1 log CFU/g (Table 2). When mixed according to manufacturer instructions for achieving 1000 ppm, Sanova® yielded chlorite ion concentrations of 414 ± 154 ppm within 30 min and 24 h. TriNova and Sanova treatments containing 200 ppm chlorite had a deleterious effect on product quality, with noticeable bleaching at the cut edges. Both untreated and treated samples of lettuce had large numbers of injured *E. coli* O157:H7 (Tables 1–4).

3.2. Impact of bacterial attachment time on the effectiveness of sanitizer treatments

Efficacy of chlorine and deionized water against *E. coli* O157:H7 was not significantly affected by length of time that the microorganisms were allowed to attach to the lettuce (Table 3). All treatments of Romaine lettuce, other than deionized water, resulted in fewer injured *E. coli* O157:H7 cells following 1 d of bacterial attachment compared to the 0 d attachment.

3.3. Efficacy of acidic electrolyzed water against Escherichia coli O157:H7 on Romaine and Iceberg lettuce after various contact times

Since 2 min of treatment with AEW did not result in significant reductions of *E. coli* O157:H7, it was decided to test the efficacy of AEW after longer exposure. AEW did not result in significantly greater reductions in *E. coli* O157:H7 populations on Romaine or Iceberg lettuce at longer, as compared to shorter, contact times. Most of the AEW-induced inactivation of *E. coli* O157:H7 was achieved within 2 min (Table 4). AEW consistently resulted in <1 log CFU/g reduction even after 20 min of lettuce exposure to the treatment (Table 4).

3.4. SEM assessment of effects of sanitizer on Escherichia coli O157:H7 on Romaine lettuce

Bacterial cells, both individually and in clusters and mixed culture biofilms, were observed covering the entire surface of dip inoculated Romaine lettuce (Fig. 1A). Treatments of 20 and 200 ppm chlorine were chosen for further study by SEM to illustrate bacterial removal by washing, since 200 ppm chlorine treatment was as effective as TriNova®, Sanova®, and AEW on Romaine lettuce (Table 2), and also since these treatments are common in industry. However, the only bacteria observed on Romaine lettuce after treatment with 20 or 200 ppm chlorine were either in bacterial biofilms or infiltrating stomata (Fig. 1B) or cells grouped in small clusters (Fig. 1C). Individual bacterial cells (not in clusters or biofilms) on Romaine lettuce treated with 200 ppm chlorine were only observed to be located in damaged lettuce tissue (Fig. 1D).

4. Discussion

In the United States chlorine wash at 20–200 ppm is the most commonly used sanitizing treatment by the fresh produce industry. Alternative sanitizers to chlorine are: 1) chlorine dioxide, which is only allowable in the processing of whole, uncut fruits and vegetables as a wash at 3 ppm followed by a potable water rinse (CFR, 2008a); 2) acidified sodium chlorite, which is allowed at concentrations of 500–1200 ppm in conjunction with generally recognized as safe acids to adjust the pH to 2.3–2.9 (CFR, 2008b). To allow for comparison between the efficacies of chlorine and alternative sanitizers, commercial concentrations between 20 and 200 ppm were used to treat Iceberg and Romaine lettuce artificially inoculated with *E. coli* O157:H7.

When Sanova® was adjusted so that the chlorite ion concentration was equivalent to that of a commercial chlorine dioxide system (TriNova®), no significant differences were observed in the ability of the two solutions to inactivate *E. coli* O157:H7, except in the case

Table 4
Efficacy of acidic electrolyzed water contact time on populations of *Escherichia coli* O157:H7 on artificially inoculated lettuce stored at 4 °C for 18–24 h.

Lettuce variety	Treatment[a]	CTSMAC (log CFU/g)		Recovery (log CFU/g)		Percent injury
		Population[b]	Mean reduction	Population[b]	Mean reduction	
Iceberg	Untreated	7.19 ± 0.48[a]		7.77 ± 0.20[a]		73.7
	2 min	6.51 ± 0.56[ab]	0.68	7.05 ± 0.08[bc]	0.72	71.2
	10 min	6.69 ± 0.24[ab]	0.50	6.94 ± 0.04[c]	0.83	43.8
	20 min	6.18 ± 0.68[b]	1.01	6.89 ± 0.08[c]	0.88	80.5
Romaine	Untreated	7.18 ± 0.47[ab]		7.91 ± 0.08[a]		81.4
	2 min	6.68 ± 0.34[bc]	0.50	7.14 ± 0.17[b]	0.77	65.3
	10 min	6.99 ± 0.16[ab]	0.19	7.16 ± 0.08[b]	0.75	32.4
	20 min	6.83 ± 0.13[bc]	0.35	7.04 ± 0.05[bc]	0.87	38.3

[a] Untreated control, $n = 19$; treatments of 2 min, 10 min and 20 min, $n = 3$.
[b] Within the same column, means of the same lettuce variety not followed by the same letter are significantly different ($P < 0.05$). Data is reported as log CFU/g ± standard deviation.

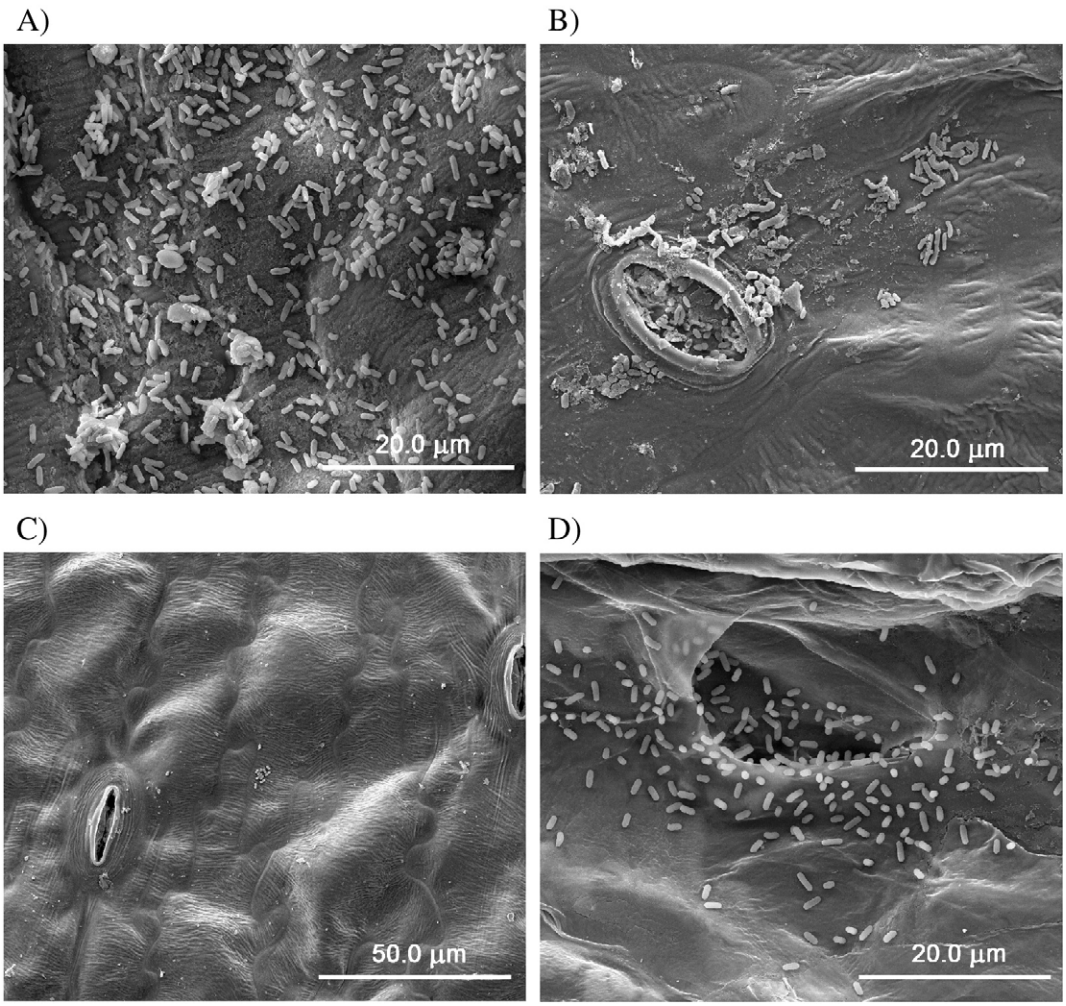

Fig. 1. Scanning Electron Microscopy micrograph of *Escherichia coli* O157:H7 on Romaine lettuce inoculated with the pathogen, stored at 4±2 °C for 24 h then treated under the following conditions: A) no wash treatment, B) *E. coli* O157:H7 in biofilms after 2 min wash with 20 ppm chlorine, C) small cluster of *E. coli* O157:H7 on lettuce surface after 2 min wash with 200 ppm chlorine, D) *E. coli* O157:H7 in damaged lettuce tissue after 2 min wash with 200 ppm chlorine.

of Romaine lettuce. The TriNova® solution at 200 ppm was the only sanitizer capable of reducing *E. coli* O157:H7 by more than 1 log CFU/g on Romaine lettuce. Treatments with TriNova® and Sanova® containing 200 ppm chlorite had deleterious effects on lettuce quality, with noticeable discoloration of the leaves. Aside from TriNova®, chlorine (200 ppm), AEW, and all other TriNova® and Sanova® treatments were statistically similar in their ability to reduce *E. coli* O157:H7 populations on Romaine lettuce. Similarly, Beuchat et al. (2004) reported that chlorine and peroxyacetic acid washes were less effective against *L. monocytogenes* on Romaine lettuce as compared to Iceberg.

In this study, no significant difference was found between the effectiveness of Sanova® within 30 min as opposed to Sanova® that had been used beyond this time limit. Studies that have evaluated the efficacy of Sanova® report the concentration of Sanova®, rather than the concentration of chlorite ion present in the wash solution (Gonzalez et al., 2004; Martinez-Sanchez et al., 2006; Stopforth et al., 2008). In this study we found that 1000 ppm Sanova® contained approximately 414 ppm chlorite ion, which we used to standardize the concentration of active ingredients in the solution as compared to the chlorite ion concentration of TriNova® solution.

Despite the fact that acidified sodium chlorite is allowed to be used at such high levels on foods, the most effective levels tested in this study were not significantly better than a 20 ppm chlorine solution in reducing *E. coli* O157:H7 populations on Romaine lettuce. The highest level tested here, which was well below 1000 ppm Sanova® (200 ppm chlorite ion) resulted in noticeable discoloration of both Romaine and Iceberg lettuce. On Iceberg lettuce, 100 ppm chlorite in Sanova® did result in a significant reduction in *E. coli* O157:H7 without such noticeable discoloration, but it was not significantly better than AEW. The most effective treatment against *E. coli* O157:H7 on both types of lettuce was 200 ppm chlorite ion, which produced noticeable discoloration of both varieties of lettuce. Under the conditions used in this experiment, populations of *E. coli* O157:H7 on Romaine lettuce could not be reduced by more than 90%, which is less than the population reductions reported in studies of sanitizer efficacy against spray- or spot-inoculated produce (Gonzalez et al., 2004; Martinez-Sanchez et al., 2006; Park et al., 2008; Stopforth et al., 2008).

For most product and treatment combinations, higher numbers of injured bacteria were detected on the day of inoculation as opposed to the following day, possibly due to the effect of desiccation on the lettuce leaves during the inoculation procedure. It is possible that the cells that were injured on the day of inoculation were either already dead or were more susceptible to inactivation by the various treatments the following day, resulting in the detection of fewer injured cells.

As with the results reported here, AEW and chlorine (200 ppm) have been found by other researchers to achieve less than 1 log CFU/g reduction of *E. coli* O157:H7 on head lettuce within 1 min of washing (Koseki et al., 2003), whereas other investigators have reported in

excess of 2 log CFU/g reduction of *E. coli* O157:H7 on spray-inoculated leafy greens within the same treatment time (Park et al., 2008; Stopforth et al., 2008). Venkitanarayanan et al. (1999) reported that AEW treatment of *E. coli* O157:H7 cells in liquid suspension resulted in 7 log CFU/ml reduction within 5 min of exposure and complete inactivation within 10 min. Experiments were conducted within this study to test for inactivation *E. coli* O157:H7 on lettuce after 10 and 20 min. However, most of the population reduction was achieved within the first 2 min of treatment with AEW. Similar tests of extended chlorine treatment times have also found that the majority of the population reduction occurs within the first 2 min of treatment (Akbas and Olmez, 2007; Beuchat, 1999).

Other studies have reported that the pathogen reductions achieved depended on the inoculation method, with the greatest log reductions being observed on samples that were spot- or spray-inoculated (Beuchat, 1999; Beuchat et al., 2004; Burnett et al., 2004; Koseki et al., 2003; Lang et al., 2004). Confocal scanning laser microscopy studies have shown that in dip-inoculated lettuce the bacterial cells were located in infiltrating stomata, trichomes, cut edges, and damaged tissue of lettuce, and that the bacteria in these areas are less accessible to sanitizers (Seo and Frank, 1999). In contrast, the bacteria that were present on the intact lettuce leaf surface were largely killed by sanitizing with chlorine (Takeuchi and Frank, 2000). Similar results were obtained in this study by using SEM, with lettuce washed with 20 and 200 ppm of chlorine showing markedly fewer cells scattered along the undamaged surface. Following a 2 min wash in 200 ppm chlorine, cells matching the morphology of *E. coli* O157:H7 were still present in damaged lettuce tissue, in stomata, and incorporated into mixed culture biofilms containing filamentous fungi and yeasts following a 2 min wash in 200 ppm chlorine. These areas provide harborage sites and, thus, protection to *E. coli* O157:H7 from sanitizers (Annous et al., 2006, 2009). This is a cause for concern, since it has been shown that damaged and cut lettuce surfaces provide substrates to allow for subsequent *E. coli* O157:H7 proliferation in these areas (Brandl, 2008).

Results from this study indicated that major factors which are important in limiting the efficacy of sanitation treatments of lettuce are the attachment of pathogenic cells to inaccessible sites on the surface of lettuce and/or the incorporation of those cells within biofilms in such inaccessible sites (Annous et al., 2006, 2009). Therefore, the development of new technologies, capable of improving the exposure of potential pathogens' harborage sites on lettuce to sanitizing agents, is required.

Although sanitation treatments of lettuce using water generally were not significantly different from wash with sanitizing agents, it is recommended that sanitizing agents are used during all wash treatments of fresh produce including lettuce. The use of a sanitizing agent during wash treatment would eliminate the microbial load in the washing solution and thus prevent any possible cross contamination in the washing tank.

Acknowledgements

This work was funded by USDA-CSREES Grant Number 2006-51110-03681. The authors thank Dr. Peter H. Cooke for the scanning electron microscopy, Mr. Paul Pierlott for his work on the micrographs, and Dr. Joshua Gurtler for his assistance with the statistical analysis, and Dr. Joshua Gurtler and Dr. Ching-Hsing Liao for their helpful comments on the manuscript. The authors also thank ICA Tri-Nova, LLC; Ecolab; and Hoshizaki Electric Co, Ltd. for providing their products for evaluation.

References

Akbas, M.Y., Olmez, H., 2007. Inactivation of *Escherichia coli* and *Listeria monocytogenes* on Iceberg lettuce by dip wash treatments with organic acids. Letters in Applied Microbiology 44, 619–624.

Allende, A., Selma, M.V., Lopez-Galvez, F., Villaescusa, R., Gil, M.I., 2008. Role of commercial sanitizers and washing systems on epiphytic microorganisms and sensory quality of fresh-cut escarole and lettuce. Postharvest Biology and Technology 49, 155–163.

Annous, B.A., Fratamico, P.M., Smith, J.L., 2009. Quorum sensing in biofilms: why bacteria behave the way they do. J. Food Sci. 74 (1), R24–R37.

Annous, B.A., Solomon, E.B., Niemira, B.A., 2006. Biofilms on fresh produce and difficulties in decontamination. Food Quality 13, 80–84.

Beuchat, L.R., 1999. Survival of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces applied to lettuce and the effectiveness of chlorinated water as a disinfectant. Journal of Food Protection 62, 845–849.

Beuchat, L.R., Adler, B.B., Lang, M.M., 2004. Efficacy of chlorine and a peroxyacetic acid sanitizer in killing *Listeria monocytogenes* on iceberg and romaine lettuce using simulated commercial processing conditions. Journal of Food Protection 67, 1238–1242.

Brandl, M.T., 2008. Plant lesions promote the rapid multiplication of *Escherichia coli* O157:H7 on post-harvest lettuce. Applied and Environmental Microbiology 74, 5285–5289.

Burnett, A.B., Iturriaga, M.H., Escartin, E.F., Pettigrew, C.A., Beuchat, L.R., 2004. Influence of variations in methodology on populations of *Listeria monocytogenes* recovered from lettuce treated with sanitizers. Journal of Food Protection 67, 742–750.

[CDPH] California Department of Public Health, 1996. *E. coli* O157:H7 outbreak linked to Fancy Cut Farms. http://www.cdph.ca.gov/pubsforms/Documents/fdb%20eru%20Sal%20EC%20FancyCutt081996.pdf. Accessed August 8, 2008.

[CDPH] California Department of Public Health, 2002. *E. coli* O157:H7 illnesses in Washington. http://www.cdph.ca.gov/pubsforms/Documents/fdb%20eru%20Rom%20Let%20EC%20WA%202002.pdf. Accessed August 8, 2008.

[CDPH] California Department of Public Health, 2004a. Report of investigation of *E. coli* O157:H7 outbreak at San Mateo County retirement facility in October 2003. http://www.cdph.ca.gov/pubsforms/Documents/fdb%20eru%20Spnch%20EC%20San%20Mateo%20Co052004.pdf. Accessed August 8, 2008.

[CDPH] California Department of Public Health, 2004b. Investigation of pre-washed mixed bagged salad following an outbreak of *Escherichia coli* O157:H7 in San Diego and Orange County. http://www.cdph.ca.gov/pubsforms/Documents/fdb%20eru%20Sal%20EC%20SD%20OR%20Co052004.pdf. Accessed August 8, 2008.

[CDPH] California Department of Public Health, 2005. Investigation of an *Escherichia coli* O157:H7 outbreak associated with consumption of Dole Brand pre-packaged salads. http://www.cdph.ca.gov/pubsforms/Documents/fdb%20eru%20Spnch%20EC%20Dole032007wph.PDF. Accessed August 8, 2008.

[CDPH] California Department of Public Health, 2007a. Investigation of an *Escherichia coli* O157:H7 outbreak associated with Dole pre-packaged spinach. http://www.cdph.ca.gov/pubsforms/Documents/fdb%20eru%20Spnch%20EC%20Dole032007wph.PDF. Accessed August 8, 2008.

[CDPH] California Department of Public Health, 2007b. Environmental investigation of *Escherichia coli* O157:H7 outbreak associated with Taco Bell restaurants in Northeastern states. http://www.cdph.ca.gov/pubsforms/Documents/fdb%20eru%20IceLet%20TacoBell112007.pdf. Accessed August 8, 2008.

[CDPH] California Department of Public Health, 2008. Investigation of the Taco John's *Escherichia coli* O157:H7 outbreak associated with iceberg lettuce. http://www.cdph.ca.gov/pubsforms/Documents/fdb%20eru%20IceLet%20TacoJohn022008.pdf. Accessed August 8, 2008.

[CFR] United States Code of Federal Regulations, 2008a. Code of Federal Regulations, Title 21, Part 173.300. Secondary direct food additives permitted in food for human consumption: chlorine dioxide. http://cfr.vlex.com/vid/19706209. Accessed November 20, 2008.

[CFR] United States Code of Federal Regulations, 2008b. Code of Federal Regulations, Title 21, Part 173.325. Secondary direct food additives permitted in food for human consumption: acidified sodium chlorite. http://cfr.vlex.com/vid/19706225. Accessed November 20, 2008.

Escudero, M.E., Velazquez, L., Di Genaro, M.S., De Guzman, A.M.S., 1999. Effectiveness of various disinfectants in the elimination of *Yersinia enterocolitica* on fresh lettuce. Journal of Food Protection 62, 665–669.

Gonzalez, R.J., Luo, Y., Ruiz-Cruz, S., McEvoy, J.L., 2004. Efficacy of sanitizers to inactivate *Escherichia coli* O157:H7 on fresh-cut carrot shreds under simulated process water conditions. Journal of Food Protection 67, 2375–2380.

Hilborn, E.D., Mermin, J.H., Mshar, P.A., Hadler, J.L., Voetsch, A., Wojtkunski, C., Swartz, M., Mshar, R., Lambert-Fair, M., Farrar, J.A., Glynn, M.K., Slutsker, L., 1999. A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. Archives of Internal Medicine 159, 1758–1764.

Hoshizaki Electric Co., Ltd., 2003. ROX water electrolyzer. http://www.hoshizakiamerica.com/rox.asp. Accessed August 11, 2008.

Kim, C., Hung, Y.-C., Brackett, R.E., Lin, C.-S., 2003. Efficacy of electrolyzed oxidizing water in inactivating *Salmonella* on alfalfa seeds and sprouts. Journal Food Protection 66, 208–214.

Koseki, S., Yoshida, K., Kamitani, Y., Itoh, K., 2003. Influence of inoculation method, spot inoculation site, and inoculation size on the efficacy of acidic electrolyzed water against pathogens on lettuce. Journal of Food Protection 66, 2010–2016.

Kross, R.D., Kemp, G.K., 2000. Method for optimizing the efficacy of chlorous acid and other disinfecting sprays for poultry and other meats. United States Patent No. 6063425.

Lang, M.M., Harris, L.J., Beuchat, L.R., 2004. Survival and recovery of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on lettuce and parsley as affected by method of inoculation, time between inoculation and analysis, and treatment with chlorinated water. Journal of Food Protection 67, 1092–1103.

Martinez-Sanchez, A., Allende, A., Bennett, R.N., Ferreres, F., Gil, M.I., 2006. Microbial, nutritional and sensory quality of rocket leaves as affected by different sanitizers. Postharvest Biology and Technology 42, 86–97.

[MMWR] Morbidity and Mortality Weekly Report, 2006. Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach—United States, September 2006, vol. 55, pp. 1045–1046. mmwr.

Nthenge, A.K., Weese, J.S., Carter, M., Wei, C., Huang, T., 2007. Efficacy of gamma radiation and aqueous chlorine on *Escherichia coli* O157:H7 in hydroponically grown lettuce plants. Journal of Food Protection 70, 748–752.

Parish, M.E., Beuchat, L.R., Suslow, T.V., Harris, L.J., Garrett, E.H., Farber, J.N., Busta, F.F., 2003. Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. Comprehensive Reviews in Food Science and Food Safety 2, 161–173.

Park, E.J., Alexander, E., Taylor, G.A., Costa, R., Kang, D.H., 2008. Effect of electrolyzed water for reduction of foodborne pathogens on lettuce and spinach. Journal of Food Science 73, M268–M272.

Rodgers, S.L., Cash, J.N., Siddiq, M., Ryser, E.T., 2004. A comparison of different chemical sanitizers for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes* in solution and on apples, lettuce, strawberries, and cantaloupe. Journal of Food Protection 67, 721–731.

Seo, K.H., Frank, J.F., 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. Journal of Food Protection 62, 3–9.

Soderstrom, A., Osterberg, P., Lindqvist, A., Jonsson, B., Lindberg, A., Ulander, S.B., Welinder-Olsson, C., Lofdahl, S., Kaijser, B., De Jong, B., Kuhlmann-Berenzon, S., Boqvist, S., Eriksson, E., Szanto, E., Andersson, S., Allestam, G., Hedenstrom, I., Muller, L.L., Andersson, Y., 2008. A large *Escherichia coli* O157 outbreak in Sweden associated with locally produced lettuce. Foodborne Pathogens and Disease 5, 339–349.

Stevens, A.A., 1982. Reaction products of chlorine dioxide. Environmental Health Perspectives 46, 101–110.

Stopforth, J.D., Mai, T., Kottapalli, B., Samadpour, M., 2008. Effect of acidified sodium chlorite, chlorine, and acidic electrolyzed water on *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* inoculated onto leafy greens. Journal of Food Protection 71, 625–628.

Takeuchi, K., Frank, J.F., 2000. Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. Journal of Food Protection 63, 434–440.

Venkitanarayanan, K.S., Ezeike, G.O., Hung, Y., Doyle, M.P., 1999. Efficacy of electrolyzed oxidizing water for inactivating *Escherichia coli* O157:H7, *Salmonella enteritidis*, and *Listeria monocytogenes*. Applied and Environmental Microbiology 65, 4276–4279.

Zhang, S., Farber, J.M., 1996. The effects of various disinfectants against *Listeria monocytogenes* on fresh-cut vegetables. Food Microbiology 13, 311–321.